PATENT
Docket No. 20-4559P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicants: Eijiro WATANABE et al.

Serial No.: 09/301,766 Group: 1638

Filed: April 29, 1999 Examiner: D.H.Kruse

For: RAFFINOSE SYNTHASE GENES AND THEIR USE

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Eijiro WATANABE, citizen of Japan and residing in Fukui-cho 32-12-403, Takarazuka-shi, Hyogo-ken, Japan, declare and say that:

1. I completed the doctor's course, with a major in agricultural chemistry, of the graduate school of Tokyo University and obtained a doctor's degree in agriculture at Tokyo University in March, 1991.

2. From April, 1991, I made further researches in the Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University, as a postdoctoral fellow (Japan Society for the Promotion of Science) for one year.

3. From April, 1992 to the present, I have been an employee of Sumitomo Chemical Company, Limited, the assignee of the above-identified application.

4. From April, 1992 to March, 2000, I had been engaged in research works for plant engineering using recombination and other gene manipulation, such as cloning of plant genes, preparation and evaluation of transgenic plants.

5. I am one of the inventors of the above-identified application and am familiar with the subject matter thereof.

6. I have read the Office Action mailed August 11, 2003 and the reference cited, and am familiar with the subject matter thereof.

7. To demonstrate successful expression of raffinose synthase activity in transgenic plants, I have made the following experiments.

Experiments

Transformation of Tobacco with Raffinose Synthase Gene Derived from Brassica Plant

The vector BjRS-Sac(+)-121 having the mustard raffinose synthase gene of the present invention in the expressible direction (*i.e.* sense direction) and the vector BjRS-Sac(-)-121 having the mustard raffinose synthase gene of the present invention in the reverse direction (*i.e.* antisense direction), which are the same as obtained in Example 8 of the present specification, were used for the transformation of tobacco (*Nicotiana tubacum*) by the Agrobacterium infection method.

Agrobacterium tumefaciens (strain LBA4404 having rifampicin and streptomycin resistance) previously converted into a competent state by calcium chloride treatment was transformed independently with two plasmids BjRS-Sac(+)-121 and BjRS-Sac(-)-121. The transformants were selected on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin by utilizing the kanamycin resistant character conferred by the kanamycin resistant gene (neomycin phosphotransferase, NPTII) of the introduced plasmids.

The transformant Agrobacterium obtained (*Agrobacterium tumefaciens* strain LBA4404, rifampicin and streptomycin resistant) was cultured on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin at 28°C for a whole day and night, and the culture was used for the transformation of tobacco by the method described below.

Seeds of tobacco were aseptically sown on 1/2 MS medium containing 2% sucrose and 0.7% agar. After one week, leaves of sprouting plants were cut out with a scalpel, and transferred to MS medium containing 3% sucrose, 0.7% agar, 1.0mg/l BA and 0.1mg/l NAA, followed by preculture for 1 day. The precultured leaves were transferred in a 1000-fold dilution of the Agrobacterium culture broth and allowed to stand for 5 minutes. The leaves were transferred again to the same medium as used in the preculture, and cultured for 3 to 4 days. The cultured leaves were transferred to MS medium containing 3% sucrose, 1.0mg/l BA, 0.1mg/l NAA and 500 mg/l cefotaxim, and shaken for 1 day to remove microbial cells. The leaves thus treated were transferred to

MS medium containing 3% sucrose, 0.7% agar, 1.0mg/l BA, 0.1mg/l NAA, 100 mg/l cefotaxim and 20 mg/l kanamycin, and cultured for 3 to 4 weeks. The leaves were transferred to MS medium containing 3% sucrose, 0.7% agar, 1.0mg/l BA, 0.1mg/l NAA, 100 mg/l cefotaxim and 20 mg/l kanamycin, and cultivated. The cultivation on this medium was continued with subculturing at intervals of 3 to 4 weeks. When shoots were began to regenerate, these shoots were subcultured on MS medium containing 3% sucrose, 0.7% agar and 20 mg/l kanamycin, and cultivated for 3 to 4 weeks. The rooting plants were transferred to vermiculite : peat moss = 1 : 1, and cultivated at 21°C to 22°C in a cycle of day/night = 12 hours : 12 hours. With the progress of plant body growth, the plants were grown with cultivation soil.

Measurement of Raffinose Synthase Activity

Leaves of the transformed tobacco plant were put in 10 times of the leaf weight of 100 mM Tris-HCl (pH 7.4), 5 mM DTT (dithiothreitol), 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM benzamide, and ground on ice with a mortar. The ground material was centrifuged at 21,400 x g for 50 minutes at 4°C. The resulting supernatant was recovered and used as a sample for the following measurement of raffinose synthase activity.

The raffinose synthase activity was measured under the following conditions according to the description of L. Lehle and W. Tanner, Eur. J. Biochem., 38, 103-110 (1973).

First, 2 μl of a sample to be used in the measurement of activity was added to 18 μl of the reaction mixture that came to contain 100 mM Tris-HCl (pH 7.4), 5 mM DTT (dithiothreitol), 0.01% BSA, 200 μM sucrose, 5 mM galactinol, 31.7 μM [^{14}C] sucrose, and the reaction mixture was kept at 37°C for 18.3 hours. After the reaction, 30 μl of ethanol was added to the reaction mixture, followed by stirring and centrifugation at 15,000 rpm for 5 minutes. The supernatant was spotted at a volume of 5 μl on an HPTLC plate of cellulose for thin layer chromatography (Merck, 10 cm x 20 cm), and developed with n-butanol : pyridine : water : acetic acid = 60 : 40 : 30 : 3. The developed plate was dried and then quantitatively analyzed with an imaging analyzer (Fuji Photographic Film, FUJIX Bio Imaging Analyzer BAS-2000II) for the

determination of content of [^{14}C] raffinose. Raffinose synthase activity in each sample was calculated from the content of [^{14}C] raffinose.

Results

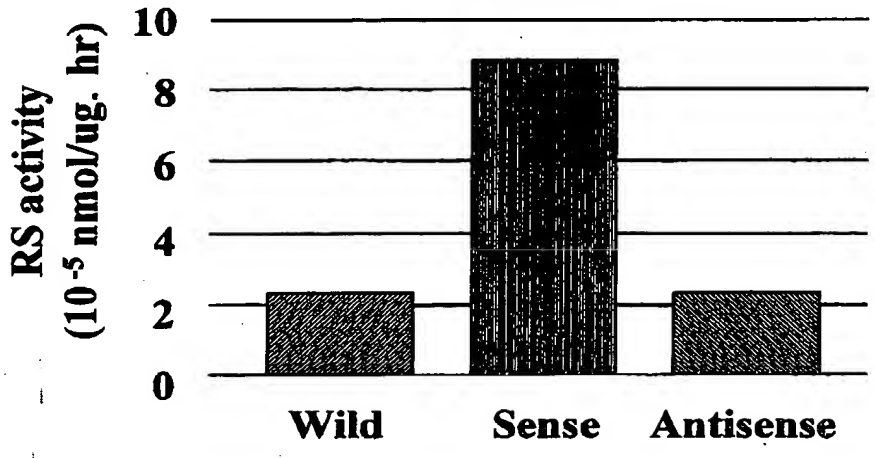
Results are summarized in Fig. 1. The transformed tobacco plant with BjRS-Sac(+)-121 ("Sense" in the figure) showed significantly higher level of raffinose synthase activity in leaf than the wild type ("Wild" in the figure).

Discussion

As can be seen from Fig. 1, the transformed tobacco plant having the mustard raffinose synthase gene of the present invention in sense direction exhibited higher raffinose synthase activity as compared with the control tobacco plant having no such gene. This indicates that tabacco plants may have improved raffinose synthase activity by introduction of the raffinose synthase gene of the present invention in sense direction into these plants.

Thus, it is clearly demonstrated that the raffinose synthase gene of the present invention can successfully express raffinose synthase activity in the transformed tobacco plant.

Fig.1



8. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonments, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

This day of February, 2004

Eijiro WATANABE